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Enhanced Uptake of Ifosfamide into GH3 Prolactinomas with Hypercapnic Hyperoxic Gases Monitored *In Vivo* by ^{31}P MRS

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Abstract

Previously, ^{31}P magnetic resonance spectroscopy (MRS) has been used to detect ifosfamide (IF) *in vivo* and to show that breathing carbogen (5% CO_2/95% O_2) enhances the uptake and increases the efficacy of IF in rat GH3 prolactinomas [Rodrigues LM, Maxwell RJ, McSheehy PMJ, Pinkerton CR, Robinson SP, Stubbs M, and Griffiths JR (1997). *In vivo* detection of ifosfamide by ^{31}P MRS in rat tumours; increased uptake and cytotoxicity induced by carbogen breathing in GH3 prolactinomas. *Br J Cancer* 75, 62–68]. We now show that other hypercapnic and/or hyperoxic (5% CO_2 in air, 2.5% CO_2 in O_2) gas mixtures also increase the uptake of IF into tumors, measured by ^{31}P MRS. All gases caused an increased uptake (C_{max}) of IF compared to air breathing, with carbogen inducing the largest increase (85% (P<.02) compared to 46% with 2.5% CO_2 in O_2 (P<.004) and 48% with 5% CO_2 in air (P<.004)). The T_{max} (time of maximum concentration in tumor posintravenous injection of IF) was significantly (P<.04) later in the cohort that breathed 5% CO_2 in air. The increased uptake of IF with carbogen breathing was selective to tumor tissue and there were no significant increases in any of the normal tissues studied, suggesting that any host tissue toxicity would be minimal. Carbogen breathing by patients causes breathlessness. There was no significant difference in IF uptake between breathing carbogen and 2.5% CO_2 in O_2 and, therefore, the ability of 2.5% CO_2 in O_2 to also increase IF uptake may be clinically useful as it causes less patient discomfort.

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Introduction

The inadequacy of tumor vasculature is a major disadvantage with regard to the efficacy of both chemotherapy and radiotherapy. In chemotherapy, where tumor blood flow is usually a vehicle for delivery of therapeutic agents, a reduced blood flow or volume within the tumor will limit the delivery of these agents. Reduced tumor oxygenation or presence of hypoxic tissue renders the tumor more resistant to radiotherapy and because oxygen is supplied to the tumor through the blood flow, a reduced blood flow or volume will reduce the efficacy of radiotherapy. The physiological factors that could contribute to the reduced therapeutic effect include a heterogeneous and immature blood supply, reduced vessel size and diameter, and greater transport distances across the interstitium. These physiological barriers limit access of drugs to many parts of the tumor both through inadequate drug uptake and nonoptimal distribution, and also render the tumor radioresistant [1].

Several physical factors (e.g., hyperthermia, radiation) and chemical modifiers (e.g., vasoactive drugs) have been used to increase tumor blood flow to enhance therapeutic response [2]. Recently, carbogen (5% CO_2/95% O_2) breathing has been shown to increase both blood oxygenation/flow [3,4] and blood volume of tumors [5], thereby increasing radiosensitivity [6] and enhancing the effectiveness of chemotherapy [7,8].

Carbogen was originally used as an adjuvant for radiotherapy to reoxygenate hypoxic tissue. The CO_2 component was incorporated with oxygen to provide a more physiological environment and also to act as a vasodilator to prevent the hyperoxic vasoconstriction of blood vessels [6,9].

Numerous studies have recently shown the effect of carbogen breathing on tumor physiology, metabolism, and therapy. In particular, noninvasive blood oxygen level–dependent magnetic resonance imaging (BOLD MRI) has been used to show increases in tumor blood oxygenation with carbogen breathing both in preclinical [3] and clinical [10,11] studies. Changes in tumor metabolism have also been reported: breathing carbogen has been shown by ^{31}P magnetic resonance spectroscopy (MRS) to change tumor pH and energy status and by non-MR methods to increase blood glucose and decrease tumor lactate [12].

^{19}F and ^{31}P MRS have been used to study the pharmacokinetics and pharmacodynamics of fluorinated drugs, e.g., 5-fluorouracil (5FU) [13], and phosphorus-containing

Abbreviations: IF, ifosfamide; MRS, magnetic resonance spectroscopy; BOLD MRI, blood oxygen level–dependent magnetic resonance imaging; T_{max}, time of maximum observed IF concentration

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drugs, e.g., ifosfamide (IF) [7], noninvasively, *in vivo*. This method has the potential of tailoring treatment to an individual patient. Carbogen breathing before and during drug administration has been shown by MRS to increase the uptake [8,14] and enhance the efficacy of 5FU [8] and IF [7] in experimental tumors, and may therefore be useful in the clinic for selectively increasing the uptake of chemotherapeutic agents into tumors [10,15]. A disadvantage, however, of breathing carbogen in the clinic is the degree of patient discomfort caused by breathing 5% CO_2, where patients are known to experience symptoms of breathlessness, probably due to the increased respiratory drive caused by the CO_2. Recent studies have shown that inhalation of 2% CO_2 in oxygen in clinical studies [16] and 2.5% CO_2 in oxygen in preclinical studies [17] improves oxygenation and radiosensitization of tumors as effectively as carbogen. Moreover, the patients breathing 2% CO_2 in oxygen were reported to be more comfortable and found breathing the gas easier. More recently, Thews et al. [4] have shown that breathing gases with high CO_2 fractions (2.5% or 5%) leads to a prolonged improvement of tumor pO_2 and blood flow after the end of inspiratory hyperoxia when compared to 100% O_2. It has also been shown by noninvasive BOLD MRI in rat GH3 prolactinomas that there are similar increases in tumor blood oxygenation with 1% and 2.5% CO_2 in O_2 to those seen with carbogen [18]. The above studies all support the concept that the levels of CO_2 in carbogen can be reduced without compromising the enhanced oxygenation, radiosensitization, and blood flow of tumor tissue. Could the same hold for the uptake of chemotherapeutic agents?

It has previously been shown by *in vivo* ^{31}P MRS that breathing carbogen increases the uptake of the prodrug IF into GH3 prolactinomas and enhances the efficacy of treatment [7]. This was thought to be due to an increase in tumor blood volume during carbogen breathing, which was probably accompanied by an increased delivery of 4-hydroxyifosfamide, the activated form of IF. Alternatively, the tumor itself may be able to activate IF *in situ* as suggested by cell culture studies [7]. The aim of this present study was, firstly, to determine whether breathing hypercapnic and/or hyperoxic gases with a lower CO_2 content would still cause an enhanced IF uptake and, secondly, to determine if the enhanced uptake of IF with carbogen was solely due to the vasodilative effects of CO_2 on tumor blood vessels.

Materials and Methods

IF (Mitoxana; ASTA Medica, Cambridge, UK) was made up freshly at a concentration of 100 mg/ml in normal saline, at pH 7.2 to 7.4, and administered intravenously at a dose of 250 mg/kg, which is equivalent to 1.5 g/m^2, using the surface law formula [19]. If the animal was to recover from the anaesthetic, MESNA (Uromitexan), a uroprotector was administered intravenously (150 mg/kg).

Animals and Tumors

GH3 prolactinomas were grown subcutaneously in the flanks of 180- to 200-g female Wistar Furth rats as previously described [20]. Tumor volume was calculated using the formula $((\pi/6)d_1d_2d_3)$ and the mean tumor volume for the MR experiments was 5.27±1.28 cm^3. All experiments were performed in accordance with the UK Home Office Animals Scientific Procedures Act 1986.

^{31}P MRS

Animals were anesthesized with pentobarbitone (40 mg/kg) and maintained at 37°C with a warm water blanket. The tail vein was cannulated and an intravenous line placed for administration of IF while the animal was in the magnet. ^{31}P MRS spectra were obtained on a 4.7T Varian Unity Inova spectrometer with a 20-mm, two-turn surface coil. Non-localized ^{31}P spectra were obtained using an adiabatic sincos pulse, TR of 3 s, and 64 or 128 acquisitions. Cohorts of animals breathed air for up to 7 minutes and then either certified gas mixtures of carbogen (n=4), 2.5% CO_2 in oxygen (n=5), or 5% CO_2 in air (n=4) at a rate of 2 l/min for 10 minutes through a mask equipped with a scavenger. IF was administered intravenously during the tenth minute of each gas regime. Air breathing was resumed after each gas regime. The control group of animals (n=3) breathed air throughout the experiment. Spectra were acquired for up to 3 hours post-IF injection.

Quantitation of Spectra and Pharmacokinetic Analysis

Spectra were quantitated using VARPRO, a time domain nonlinear least squares method [21]. The data were fitted by assuming contributions from IF, phosphomonoesters (PME), inorganic phosphate (P_i), phosphocreatine (PCr), and the α-, β-, and γ-nucleoside triphosphate (NTP) resonances. To normalize the data, the IF level was expressed as a ratio of IF to total phosphate signal, i.e., sum of PME, P_i, PCr, and α-, β-, and γ-NTP in the signals in the spectra, as total phosphate was unlikely to change over the time course of the experiments. Tumor energetic status was assessed by determining the β-NTP/P_i ratio, and pH was calculated from the chemical shift of P_i relative to α-NTP at -7.57 ppm according to Ref. [22].

The pharmacokinetic parameters of area under the curve (AUC; IF/$\sum$P *versus* time), $T_{1/2}$ (half-life [minutes] of IF in tumor), and C_{max} (maximum IF concentration by pharmacokinetic analysis) were computed from the IF/$\sum$P *versus* time (minutes) curve using the PCNONLIN (Lexington, KY) software, which provides a least squares estimate of the parameters in a nonlinear model. The best fit was obtained using a one-compartment model with bolus input and first-order output using the equation:

$$CT = D/V * \exp(-K_{el} * T)$$

where C=IF/$\sum$P, T=time (minutes), D=dose, V=volume, and K_{el}=elimination rate. This yielded values of AUC, C_{max}, and $T_{1/2}$ ($0.693/K_{el}$) for each individual tumor.

^{31}P MRS of Extracts

A separate cohort of GH3 tumor-bearing animals (n=4) was anesthesized and breathed either air or

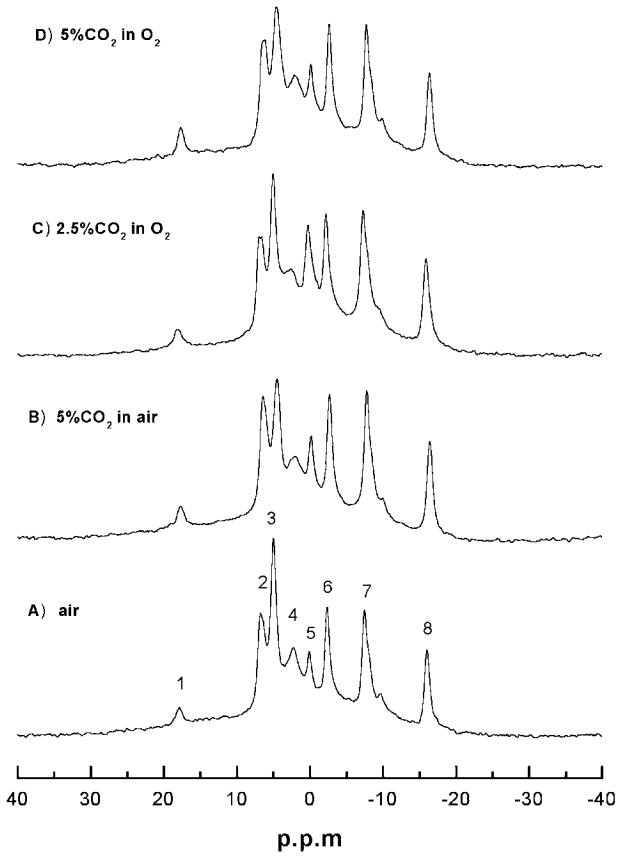

Figure 1. *Representative ^{31}P MRS spectra of GH3 prolactinomas 12 minutes after intravenous injection of IF (250 mg/kg) in rats breathing (A) air, (B) 5% CO_2 in air, (C) 2.5% CO_2 in O_2, and (D) 5% CO_2 in O_2. Peak assignments are as follows: (1) IF; (2) PME; (3) P_i; (4) phosphodiesters; (5) PCr; (6) γ-NTP; (7) α-NTP; (8) β-NTP.*

carbogen for 10 minutes. IF was injected intravenously (250 mg/kg) in the tenth minute, and after a further 15 minutes, the tumor, liver, spleen, muscle, kidney, heart, and lung were excised and freeze-clamped. The frozen tissues were ground in liquid nitrogen and extracted with cold 6% perchloric acid, centrifuged, and the supernatants neutralized. The neutralized extracts were freeze-dried and taken up in 50 mM triethanolamine/15 mM EDTA buffer containing 10% D_2O and an internal standard of 2 μmol of dimethyl methyl phosphonate (DMMP). ^{31}P MRS spectra were acquired on a Bruker AMX400 spectrometer at 162 MHz with a spectral width of 70 kHz, a 30° flip angle, 0.5 s acquisition time, 3 s relaxation delay, and 512 scans. IF levels were calculated relative to DMMP using peak integrals.

Statistical Analysis

All results were presented as mean values ±1 SEM. Comparisons between the groups were performed using the two-tailed Student's *t*-test. The significance level was set at 5%.

Results

IF resonates +18 ppm from PCr in the ^{31}P MRS spectrum. It was detected in all tumors, regardless of which gas the animal was breathing, within a few minutes of an intravenous injection (Figure 1) and was still detectable at the end of the experiment, 3 to 4 hours later.

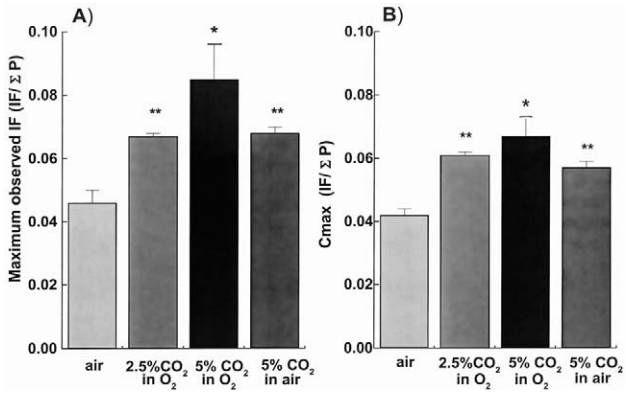

Figure 2. *Effect of breathing different gas mixtures on the (A) maximum observed IF (IF/$\sum$P) and the (B) C_{max} (maximum IF/$\sum$P from pharmacokinetic analysis). Mean ± SEM (n = 3–5). *P < .02, **P < .004 compared to air breathing.*

Effect of Carbogen, 2.5% CO_2 in Oxygen, and 5% CO_2 in Air on IF Pharmacokinetics in GH3 Tumors

Compared to air breathing, all three gas regimes caused significant increases (ranging from 50% to 70%) in the maximum observed IF concentration (expressed as a ratio of IF to total phosphate, to normalize the data) and in C_{max} (maximum IF concentration by pharmacokinetic analysis) in the tumor (Figure 2, *A* and *B*). In contrast, T_{max} (time of the maximum observed IF concentration in tissue) was significantly later with 5% CO_2 in air (22.4 ± 1.8 minutes) compared to air alone (13.9 ± 2.5 minutes, $P < .04$), but not significantly later with carbogen (12.8 ± 3.7 minutes) or 2.5% CO_2 in O_2

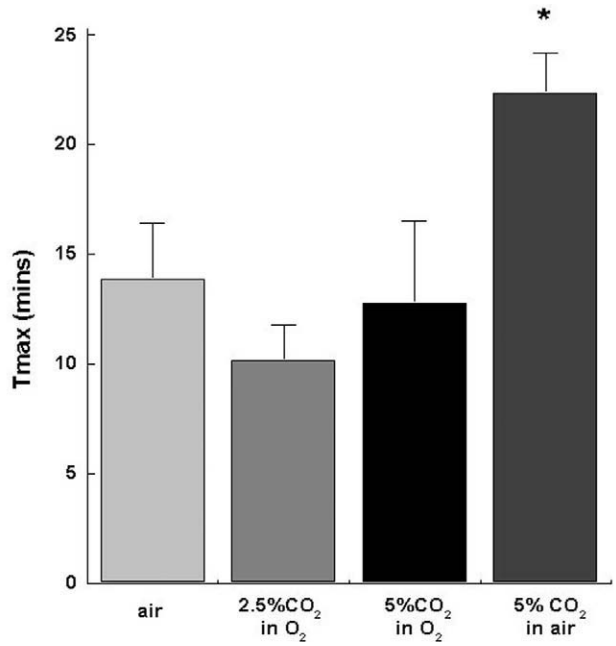

Figure 3. *Effect of breathing different gas mixtures on the T_{max} (time of maximum observed IF/$\sum$P) in tissue as observed by ^{31}P MRS. Mean ± SEM (n = 3–5). *P < .04 compared to air breathing.*

Table 1. Effect of Breathing Different Gases on IF Pharmacokinetics in the GH3 Prolactinoma Obtained from *In Vivo* ^{31}P MRS Spectra.

	$T_{1/2}$ (min)	AUC
Air	274 ± 67	16.3 ± 3.6
5% CO_2 in air	236 ± 39	19.7 ± 3.6
2.5% CO_2 in O_2	204 ± 21	18 ± 1.6
5% CO_2 in O_2	186 ± 42*	17.7 ± 3.9

Results show the mean ± SEM (n=3 or 4), where $T_{1/2}$ is the half-life for elimination and AUC is the area under (IF/ΣP) *versus* time.
*P>.1 compared to air breathing.

(10.2 ± 1.57 minutes) compared to air alone (Figure 3). Changes in the other pharmacokinetic parameters, $T_{1/2}$ and AUC, were not significantly different (P>.1) between air breathing and each of the gas regimes (Table 1). It should be noted that *in vivo* ^{31}P MRS detects only the prodrug IF and not its activated form, 4-OH IF, and therefore, the measure of C_{max} rather than AUC may be more relevant to the tumor response.

Effects of IF on Endogenous Tumor Phosphorus Metabolites and Tumor pH with the Three Gas Regimes

A time course showed that prior to injection of IF, there was a tendency for the β-NTP/P_i ratio to increase with 2.5% CO_2 in O_2 and carbogen breathing, but not with 5% CO_2 in air breathing (Figure 4). Similar observations have been previously made in GH3 prolactinomas with longer carbogen breathing times [18]. However, within 40 minutes of IF administration, β-NTP/P_i decreased with all gas regimes (P<.04 with air breathing or 2.5% CO_2 in O_2; P<.1 with carbogen or 5% CO_2 in air breathing). The minimum level of β-NTP/P_i post-IF injection was not significantly different between the gas regimes, except between 5% CO_2 in air and 2.5% CO_2 in O_2 (P=.04), which could be a consequence of a significantly longer T_{max} with 5% CO_2 in air. There was also a concomitant fall in pH of between 0.05 and 0.15 pH units with all gas regimes (P<.05 with carbogen and 5% CO_2 in air)

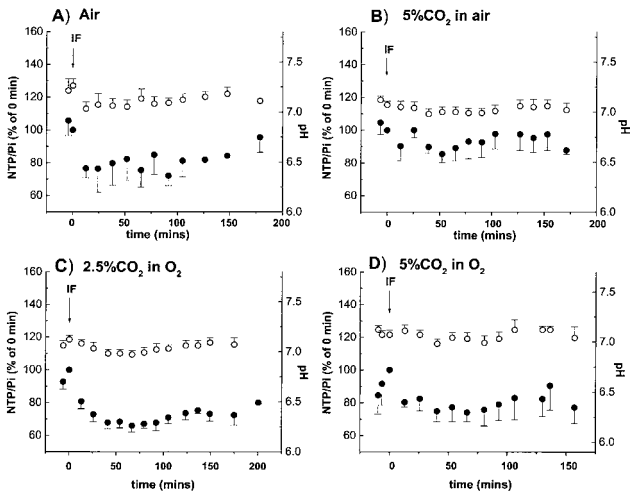

Figure 4. *Effect of intravenous IF (250 mg/kg) on β-NTP/Pi (●) and pH (○) in GH3 prolactinomas with the following breathing regimes: (A) air, (B) 5% CO_2 in air, (C) 2.5% CO_2 in O_2, (D) 5% CO_2 in O_2. Mean ± SEM (n=4 or 5).*

Table 2. Effect of Carbogen Breathing on IF Levels in Tumor and Normal Tissue Extracts, Measured by ^{31}P MRS.

Tissue	IF (μmol/g wet weight) (Air Breathing)	IF (μmol/g wet weight) (Carbogen Breathing)
Tumor	0.057 ± 0.01	0.166 ± 0.03*
Liver	0.22 ± 0.08	0.17 ± 0.08
Spleen	0.2 ± 0.02	0.17 ± 0.05
Kidney	0.26 ± 0.03	0.29 ± 0.06
Muscle	0.2 ± 0.04	0.18 ± 0.02
Lung	0.27 ± 0.07	0.22 ± 0.08
Heart	0.14	0.18 ± 0.02

Mean ± SEM (n=3 or 4, except heart [air breathing] where n=2).
*P<.02.

post-IF injection with recovery within 2 to 4 hours (Figure 4). As previously reported, this fall in β-NTP/P_i and pH is thought to be due to an acute drug-induced hypotension because the mean arterial blood pressure fell to 70% to 90% of control within 10 to 15 minutes of intravenous injection of IF [7].

Effect of Carbogen Breathing on IF Levels in Perchloric Acid Extracts of Tumor and Normal Tissue by In Vitro ^{31}P MRS

IF was detected in all tissues extracted, at a chemical shift of +18 ppm relative to PCr. Levels of IF in the control rats (air breathing) were between 0.14 and 0.27 μmol/g wet weight in all tissues except tumor, where levels were 0.06 ± 0.01 μmol/g wet weight. In tumor tissue in the rats breathing carbogen, the IF levels were two- to threefold higher at 0.17 ± 0.03 μmol/g (n=4, P<.02), whereas in all other normal tissues studied, carbogen breathing caused no significant changes (Table 2).

Discussion

These results show that all three gas regimes (carbogen, 2.5% CO_2 in O_2 and 5% CO_2 in air) caused an increase in IF uptake into the tumor, probably because hypercapnic and/or hyperoxic gas mixtures increase tumor blood volume. This is consistent with the BOLD MRI data [18], where an increase in image intensity caused by an increase in tumor oxygenation and/or blood volume was observed with all three gas regimes. In addition, the characteristic leakiness of tumor blood vessels would also facilitate drug trapping.

Breathing carbogen (5% CO_2 in O_2) caused a greater enhancement in IF uptake than either 2.5% CO_2 in O_2 or 5% CO_2 in air. Previous studies showed no differences in radiotherapeutic response between 2% [16] and 2.5% [17] CO_2 in O_2 when compared to carbogen breathing. Also, breathing either 1% CO_2, 2.5% CO_2 in oxygen, or carbogen caused responses of a similar magnitude in the transverse relaxation rate (R_2*) measured by BOLD MRI when compared to air breathing [18]. In tumors, the BOLD effect, which is dependent on the endogenous paramagnetic contrast agent, deoxyhemoglobin, is mediated by a complex combination of oxygenation, blood flow, and blood volume [5]. The lack of increase in the BOLD effect and radiosensitivity with carbogen breathing compared to 2.5% CO_2 in

oxygen breathing can be explained in the following way: with 2.5% CO_2 compared to 5% CO_2 in carbogen, the oxygen–hemoglobin dissociation curve will be shifted to the left, thereby increasing the amount of oxygen bound to the hemoglobin. This increased oxygen supply and lower fraction of deoxyhemoglobin to the tumor capillary bed might compensate for the reduced blood flow and blood volume with lower (2.5%) CO_2, leaving the BOLD effect and radiosensitivity unchanged. However, in the present study, the amount of drug "trapped" in the tumor would merely depend on the fraction of CO_2 in the gas mixture and the degree of vasodilation and increase in blood volume that it causes [5]. In the case of carbogen breathing, the oxygen could cause vasoconstriction of normal tissue vasculature but possibly not of tumor vessels, as the latter characteristically lack smooth muscle. The resistance of tumor vasculature would decrease, increasing the blood volume and blood flow [4] through the tumor tissue. Indeed, IF levels measured in tissue extracts of normal tissue (i.e., liver, spleen, muscle, lung) were lower during carbogen breathing compared to air breathing. When breathing 5% CO_2 in air (20% oxygen), hypercapnia induces systemic vasodilation in both normal and tumor tissue. This could explain why the drug peaks at a significantly later time in the tumor with the 5% CO_2/95% air regime than with the hyperoxic gas regimes in which the vasoconstrictive effect of raised pO_2 might compensate for hypercapnia-induced vasodilation.

In conclusion, previous studies have shown that addition of CO_2 to hyperoxic gases has a beneficial effect with regard to tumor oxygenation and tumor blood flow and the levels of CO_2 in carbogen gas mixtures can be reduced without compromising the effect on oxygenation or radiosensitivity of tumors or on the BOLD MRI changes. This study shows that breathing high-content CO_2 gases is suitable for increasing the delivery of chemotherapeutic agents and although breathing carbogen caused the largest increase in uptake of IF, it was not significantly different from 2.5% CO_2 in O_2. Therefore, the ability of 2.5% CO_2 to enhance drug uptake to the patient may be clinically useful as it causes less discomfort (e.g., breathlessness) than 5% CO_2, and thus, patients' tolerance may be increased.

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